SMB

Bifidogenic Peptides

The present invention relates to bifidogenic peptides, a process for their preparation and the use of said bifidogenic peptides.

Milk is known to promote the health of infants. This is often attributed to the influence of milk on the formation of an infant-typical intestinal flora of which more than 90% consists of *Bifidobacterium bifidum*.

It has been the object of the present invention to provide peptides which have a positive influence on the intestinal flora.

This object is achieved by peptides having the features of claim 1. The peptides according to the invention are peptides obtainable by

- adding proteases to cow's milk or human milk, followed by incubation for two hours;
- centrifugation to remove milk fat;
- acidification to a pH of 2.0 with strong acids;
- removing the precipitated proteins;
- application of at least one reverse phase HPLC step;
- application of a cation-exchange HPLC step;

- collecting fractions;

- adjusting the fractions to a salt content of < 25 mM by dialysis or reverse phase HPLC for performing activity tests;

- culturing *Bifidobacterium bifidum* and *E. coli* in the presence of the fractions and selecting fractions which meet the requirement:

$$\frac{BW}{B0} - \frac{EW}{E0} \geq 0.15 \text{ (bifidogenic)}$$

wherein BW represents the germ count obtained upon 16 hours of incubation of *Bifidobacterium bifidum* in 50% Elliker broth in the presence of the peptides in a concentration of 200 µg/ml;

B0 represents the germ count obtained in the control incubation without active substances;

EW represents the germ count obtained upon 16 hours of incubation of *E. coli* in 3 g/l tryptic soy broth in the presence of the peptides in a concentration of 200 µg/ml;

E0 represents the germ count obtained in the control incubation without active substances;

- isolation of the peptide contained in this fraction;

and the amidated, acetylated, sulfated, phosphorylated, glycosylated, oxidized derivatives or fragments thereof having bifidogenic properties.

The peptides according to the invention have an antimicrobial effect against bacteria which do not occur, or only so in small amounts, in the natural

infantile intestinal flora, and they promote the growth of desired bacteria, such as bifidobacteria, by promoting the growth of bifidobacteria more than that of other bacteria or by selectively inhibiting the undesired bacteria. This property of providing bifidobacteria with an advantage with respect to growth is called "bifidogenic".

Preferably, peptides are used which have the following amino acid sequence (SEQ ID NO: 1-24, respectively in order of appearance):

R_1-EQLLRLKK-R_2, R_1-YLEQLLRLKKY-R_2, R_1-NRQRNILR-R_2,
R_1-YMNGMNRQRNILR-R_2, R_1-FQWQRNMRK-R_2, R_1-HTGLRRTA-R_2,
R_1-FTAIQNLRK-R_2, R_1-EVAARARVVW-R_2, R_1-WQRNMRKV-R_2,
R_1-LARTLKRLK-R_2, R_1-YKQKVEKV-R_2, R_1-LVRYTKKV-R_2,
R_1-KYLYEIARR-R_2, R_1-ARRARVVWCAVG-R_2, R_1-ARRARVVWCAVGE-R_2,
R_3-CIAL-R_4 R_3-CIAL-R_4

R_1-YQRRPAIAINNPYVPRTYYANPAVVRPHAQIPQRQYLPNSHPPTVVRRPNLHPSF-R_2,
R_1-GRRRRSVQWCTVSQPEATKCFQWQRNMRRVRGPPVSCIKRDSPIQCIQA-R_2,
R_1-GRRRSVQWCAVSQPEATKCFQWQRNMRKVRGPPVSCIKRDSPIQCIQA-R_2,
R_1-GRRRRSVQWCAVSQPEATKCFQWQRNMRKVRGPPVSCIKRDSPIQCIQA-R_2,
R_1-VYQHQKAMPKPWIQPKTKVIPYVRYL-R_2, R_1-ARRARVVWAAVG-R_2,
R_1-CAVGGGCIAL-R_2,
R_1-RHTRKYWCRQGARGGCITL-R_2.

wherein

R_1, R_3 independently represent NH_2, an amino acid or a peptide containing up to 100 amino acids; and

R_2, R_4 independently represent COOH, $CONH_2$, an amino acid or a peptide containing up to 100 amino acids;

and the amidated, acetylated, sulfated, phosphorylated, glycosylated, oxidized derivatives or fragments thereof having bifidogenic properties.

Preferably, R_1, R_2, R_3 and R_4 have a length of up to 50, more preferably up to 20 and most preferably up to 10 amino acids.

The peptides according to the invention can be obtained by isolation and purification from cow's milk or human milk. Alternatively, they may also be expressed in genetically engineered organisms or prepared by chemical peptide synthesis.

Another aspect of the invention is the nucleic acids coding for the bifidogenic bacteria and antibodies directed against bifidogenic peptides.

The peptides and/or nucleic acids according to the invention can be contained in medicaments together with pharmaceutically acceptable excipients. In this case, those galenic formulations and dosage forms are selected in which the peptides reach their site of action undegraded.

Preferably, the peptides according to the invention are employed in amounts of from 0.1 to 100 mg per kg of body weight. Effective amounts of nucleic acids are, for example, from 0.01 mg to 100 mg per kg of body weight. Preferably, this amount is within a range of from 1 to 10 mg per kg of body weight for the peptides and nucleic acids.

The peptides according to the invention may also be contained in foods together with nutrients.

In addition, the peptides according to the invention and/or the antibodies directed against the peptides may also be contained in diagnostic agents together with other auxiliary agents.

The peptides and nucleic acids according to the invention are suitable for the treatment of diseases caused by misplaced microbial colonizations, such as infections, inflammations, microbially induced tumors, microbially caused

degenerative diseases, diarrheic diseases, colics, deviations in the oral, intestinal and vaginal floras, caries. The misplaced microbial colonization may be caused, for example, by bacteria, fungi, yeasts, protists, viruses, mycoplasmas, filariae and/or plasmodiums.

The peptides according to the invention are also suitable as auxiliary agents in the food preparation in terms of fermentations aids.

In particular, two or more peptides are preferably used in common, or peptides are used which have two or more of the peptide sequences according to the invention. When resistances of microorganisms occur, the different ranges of activity of the individual substances or of the substances having individual sequences of the peptides according to the invention allow to achieve an optimum inhibition of the undesired microorganisms through an appropriate combination of sequences or through a combination of individual substances.

The following Examples are intended to further illustrate the invention:

Example 1

Treatment of milk

To human milk, after having been adjusted to pH 3.5 with HCl, was added pepsin (20 mg per g of protein). The enzymatic reaction was incubated at 37 °C for two hours, and stopped by five minutes of boiling. This was followed by centrifugation (20 min, 60,000 g at 4 °C) and skimming off of the milk fat. To the resultant solution was added 0.1% TFA, and centrifugation was again performed to separate off precipitated high molecular weight proteins.

HPLC purification of a bifidogenic peptide from milk

For the purification of bifidogenic peptides from milk, several HPLC separation methods have to be combined in order to achieve preparation in as high a purity as possible through an optimum separation efficiency and to separate off inactive, undesired components. The respective samples formed after each separation step must be tested in two test systems, i.e., a growth test with bifidobacteria in combination with a growth test with *E. coli* as a target (see Examples 3 and 4). For the purification, it is necessary to combine at least one reverse phase chromatographic step (preferably two reverse phase chromatographic steps) with a cation-exchange HPLC separation. In the biotests, the respective sample must be employed in a salt-poor condition in order to obtain as optimal a screening result as possible.

The first separation step was performed by means of a Parcosil C18 column (1 x 12.5 cm, 100 Å, Biotek, Heidelberg, Germany).

Buffer A: 0.1% TFA
Buffer B: acetonitrile with 0.1% TFA
Gradient: 0 to 60% B in 45 minutes
Flow rate: 2 ml/min

Detection at 280 nm (see Figure 1)

Rechromatography of fraction 23 with the same column and a more gently rising gradient (see picture):

Buffer A: 0.1% TFA
Buffer B: acetonitrile with 0.1% TFA
Gradient: 0 to 20% B in 5 minutes
20 to 50% B in 45 minutes

Detection at 214 nm (see Figure 2)

Rechromatography of fraction 16 from the preceding separation step with the same column, but another eluent, in order to change the selectivity in the separation:

Buffer A: 0.1% TFA
Buffer B: 0.1% TFA in methanol
Gradient: 0 to 40% B in 5 minutes
40 to 70% B in 45 minutes

Detection at 214 nm (see Figure 3)

Rechromatography of the active fraction 21 by cation-exchange HPLC:

Column: Parcosil Pepkat, 4 x 50 mm, 300 Å, 5 µm, Biotek, Heidelberg
Buffer A: 10 mM phosphate buffer, pH 4.5
Buffer B: buffer A with 1 M NaCl
Flow rate: 0.75 ml/min
Gradient: 0 to 15% B in 5 minutes
15 to 50% B in 35 minutes

Detection at 214 nm (see Figure 4)

Each of the fractions obtained was separately desalted in a brief reverse phase HPLC run prior to being passed to the test for antimicrobial and bifidogenic activities.

The following peptides were identified by mass spectrometry and amino acid sequencing:

Fraction 9 contained the pure bifidogenic component (SEQ ID NO: 17):

YQRRPAIAINNPYVPRTYYANPAVVRPHAQIPQRQYLPNSHPPTVVRRPNLHPSF

(casein K-63-117);

fraction 10 contained the bifidogenic component (SEQ ID NO: 19):

GRRRRSVQWCAVSQPEATKCFQWQRNMRKVRGPPVSCIKRDSPICCIQA

(neutrophile lactoferrin-20-67);

and fraction 11 contains the bifidogenic component with an adduct mass of +16, which indicates that it is an oxidation product (probably, one methionine has been oxidized).

Both peptides and the oxidation product exhibit bifidogenic activity.

Example 2

Demonstration of the growth-regulating activity on E. coli

Fractions from the HPLC were employed with *E. coli* K12. The test is performed in 3 g/l tryptic soy broth (Sigma) as follows:

For each assay, cultures of *E. coli* K12 were freshly inoculated in tryptic soy broth (Sigma, Deisenhofen, Germany, order No. T8907) (Difco Manual, 10th ed., p. 1027). The incubation of these bacteria was always performed under aerobic conditions at 37 °C for 16 hours. Peptides to be tested were given to a test solution consisting of 200 µl of 3 g/l tryptic soy broth in 96-well cell culture plates, and inoculated with 20 µl of a diluted bacterial suspension. The photometric absorption of the inoculum was 0.05, meas-

ured at 500 nm. The growth of the bacteria under the influence of the peptides was also photometrically determined in an ELISA reader after 16 hours and manually determined by microscopy.

Example 3

Demonstration of the growth-regulating activity on Bifidobacterium bifidum

For each assay, cultures of *Bifidobacterium bifidum* ATCC 29521 were freshly inoculated in Elliker broth (Difco, Detroit, USA) (tryptone 20 g, yeast extract 5 g, gelatin 2.5 g, dextrose 5 g, lactose 5 g, saccharose 5 g, sodium chloride 4 g, sodium acetate 1.5 g, ascorbic acid 0.5 g). The incubation of these bacteria was always performed under anaerobic conditions at 37 °C for 16 to 18 hours. Peptides to be tested were given to a test solution consisting of 200 µl of 50% Elliker broth in 96-well cell culture plates, and inoculated with 20 µl of a diluted bacterial suspension. The photometric absorption of the inoculum was 0.05, measured at 550 nm. The growth of the bacteria under the influence of the peptides was also photometrically determined in an ELISA reader after 16 hours and manually determined by microscopy. N-Acetylglucosamine served as a positive control. Only bifidus cultures which respond to N-acetylglucosamine can be used for this test. After some passages, bifidobacteria lose this property; in this case, they can no longer be used for this growth test.

Example 4

Those fractions in which the value

$$\frac{BW}{B0} - \frac{EW}{E0} \geq 0.15 \text{ (bifidogenic)}$$

were identified as being bifidogenic.